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MINIREVIEW

Polysaccharide Antigens of the Capsule of *Cryptococcus neoformans*

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INTRODUCTION

Cryptococcus neoformans is an opportunistic pathogen. Although the healthy rarely become infected, individuals with weakened immune systems are victimized by encapsulated forms of this organism (41). The polysaccharide capsule not only holds the secrets to the yeast's virulence (5, 36, 57) but also defines the organism's serotype specificities (2). Indeed, there is a growing body of research that describes the association of a particular serotype with disseminated forms of cryptococcosis among immunocompromised individuals (42, 60). Cryptococcosis is the fourth leading cause of death among patients with full-blown AIDS (58), and *C. neoformans* serotype A has been isolated from virtually 100% of these victims (3, 34, 56, 62).

The correlation between virulence and serotype may be related to the chemical structure of the major polysaccharide antigen of its capsule, glucuronoxylomannan (GXM). To date, cryptococcal serotypes have been defined by the immunological specificities of reciprocally absorbed polyclonal antisera (23, 29, 30). However, current research has indicated microheterogeneity in the chemical structures of several cryptococcal serotypes (11, 68, 71). These molecular differences may account for the range of virulence that has been reported for the different strains of cryptococci. Complement activation, phagocytosis, immune tolerance, cytokine activation, cellular and humoral immune responses, and potentiation of human immunodeficiency virus (HIV) infection of peripheral blood lymphocytes may all be influenced by the structure of the polysaccharide capsule (57). This review attempts to describe the current understanding of the biochemical nature of serotype specificities of *C. neoformans* and how this information may be used in more precise investigations of the pathology of cryptococcosis.

AN ECOLOGICAL PERSPECTIVE

C. neoformans is an encapsulated and potentially pathogenic yeast that is ubiquitous in our environment. The capsule is a prominent virulence factor because it is tolerogenic and antiphagocytic (4, 5, 37, 38, 50). This characteristic is also evident in the fact that acapsular mutants possess reduced virulence (6, 24). Nevertheless, the capsule probably did not evolve for its role in pathogenesis. *C. neoformans* is not an obligate animal pathogen; it thrives freely living in the environment. Under normal physiological conditions in which nitrogen, carbohydrates, and moisture are readily available, the organism flourishes, but its capsule synthesis is largely repressed (25). However, the yeast is sensitive to threatening environmental changes, e.g., decreasing concentrations of available moisture or nitrogen. Such ambient stimuli induce an increase in capsule synthesis by the yeast. In an arid environment, the capsule collapses and seals the cell with a thin cellophane-like layer of polysaccharide. In this state, the capsule, which is normally hydrophilic, provides a water barrier that protects the yeast from dehydration. In addition, with its reduced size the cell becomes small enough to penetrate the microenvironments of the lungs (55). There, the capsule layer can rehydrate and swell to near its original volume. In this form, it regains its potential to modulate the immune response. The capsule's influence on the mode of the host's immune response is manifested not only in its size but also in its chemical structure. Strain differences in virulence and pathogenicity of *C. neoformans* may be best understood by the results of ongoing and future investigations at the molecular level.

BIOCHEMISTRY OF THE CAPSULAR POLYSACCHARIDE

The cell envelope of *C. neoformans* is composed of a rigid cell wall, composed mainly of glucans (33); a capsular polysaccharide, GXM, consisting of mannose, xylose, glucuronic acid, and *O*-acetyl (2, 7, 8, 12, 68); and at least two minor carbohydrate antigens, galactoxylomannan (GalXM) and mannoprotein (MP) (13, 70). GXM, a viscous polysaccharide, and GalXM and MP together, constitute about 88 and 12%, respectively, of the capsule mass that is sloughed into the medium. GXM, GalXM, and MP are isolated from growth medium by selective precipitation with ethanol and differential complexation with hexadecyltrimethylammonium bromide (9, 12, 68). All three antigens are serologically distinct, and at least two of these components, GXM and MP, have separate effects on the immune system (54).

(i) GXM. The antigenic basis for serotype specificity is a set of structurally related capsular polysaccharides (GXM) (2, 68). The inference that the capsular polysaccharide governs serotype specificity was deduced from the observation that acapsular mutants are untypeable (31, 36). Monoclonal antibodies, produced in response to pure GXM or its conjugate, react specifically with GXM in dot-enzyme immunoassay and enzyme immunoassay (20, 22, 67). These data substantiate the role GXM plays in conferring serotype specificity on a particular *C. neoformans* isolate. Current models of GXM depict a general structure consisting of a (1→3)-linked linear α-D-mannopyranan bearing β-D-xylopyranosyl (Xyl*p*), β-D-glucopyranosyluronic acid (Glc*p*A), and 6-*O*-acetyl substituents (2, 68). A simple structural relationship between the polysaccharides of the four serotypes exists. They are all composed of a core repeating unit,

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β-D-Glc*p*A

1

↓

2

→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→

to which 2-*O*-β-D-Xyl*p* and 4-*O*-β-D-Xyl*p* units are added in increments of one to four residues. Serotypes A and D GXM are mainly substituted at *O*-2, whereas serotypes B and C GXM are substituted with Xyl*p* at *O*-2 and at *O*-4. In this way, precise molar ratios of Xyl:Man:GlcA in serotypes D, A, B, and C have been assigned as 1:3:1, 2:3:1, 3:3:1, and 4:3:1, respectively (2). Additional chemical studies of a large number of GXMs from serotypes A (17 isolates), B (6 isolates), C (5 isolates), and D (5 isolates) have been performed by methylation analysis and nuclear magnetic resonance spectroscopy (8, 10, 68, 69, 71). Detailed structural models for the GXMs of the four serotypes have been formulated on the basis of these data (Fig. 1 and 2). The linkages of the carbohydrate constituents and the sequence of these constituents have been assigned (63, 64). The data show that the molar ratio and substitution patterns proposed in the original models of GXM structure are an oversimplification, except in the case of serotype B (69). In addition, substituent dispositions characteristic of one sero-

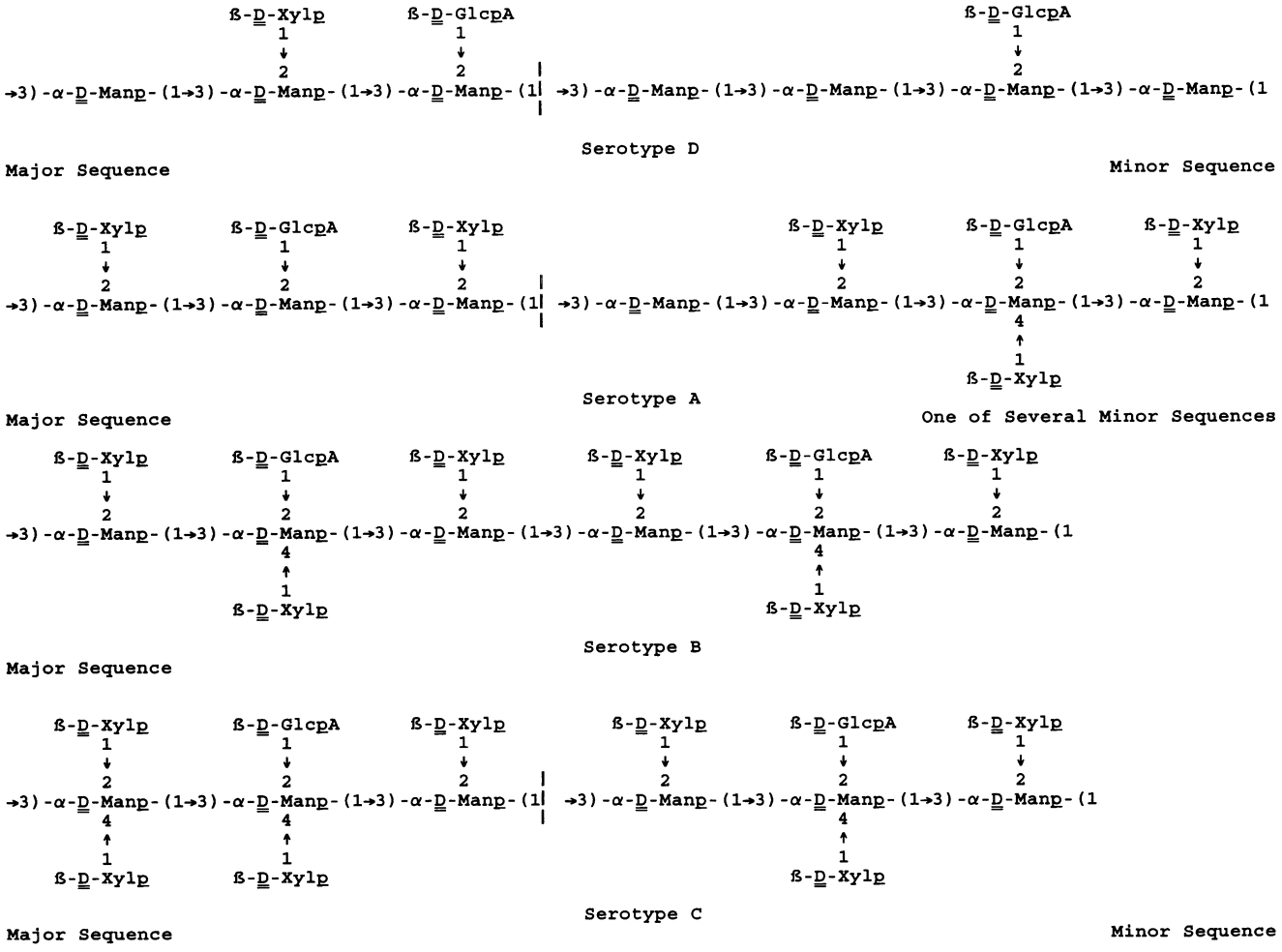

FIG. 1. Structures of the *O*-deacetylated GXMs of *C. neoformans*. The first three mannose residues represent the major repeat unit found in each of the serotypes. Structures for serotypes come from references as follows: serotype D, references 11 and 63; serotype A, reference 71; serotype B, references 64 and 69; serotype C, reference 10.

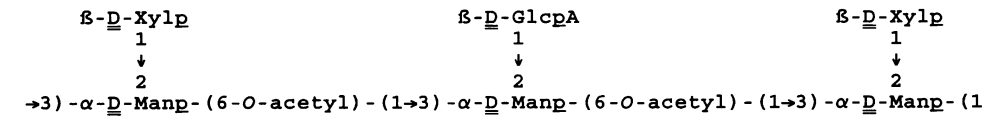

FIG. 2. Location of the *O*-acetyl groups in serotype A GXM. The structure is from an unpublished observation.

type have been identified in heterologous isolates (8, 10, 11, 68, 71).

These patterns of structural heterogeneity have inspired more sophisticated epidemiological investigations. In one study, the GXMs from 11 serotype A cultures were subdivided into four groups (71). Six isolates had almost identical substitution patterns in their GXMs; they were designated Group I. The five remaining isolates had GXMs more heterogeneous in structure than Group I; they were assigned to the remaining three groups.

(ii) MP. MP is recovered from culture filtrates and is also detected after cell wall cleavage by mechanical methods (13, 32, 70). The MP is immunogenic in rabbits, and antibodies reactive to MP have been detected in human sera (59). The MP is the antigen responsible for the delayed-type hypersensitivity (DTH) (54) observed previously with a composite antigen obtained from culture filtrates (52). MP is composed mainly of Man although significant amounts of Gal and Xyl are present, also. At least some of the Man residues are reactive with concanavalin A. This affinity has been used in the fractionation of MP since GXM and GalXM do not complex with the lectin (70). MP diffuses slowly through the dense inner layer of the wall but migrates more rapidly when it confronts the less dense outer portion of the wall. This view is consistent with the asymmetric distribution of MP in the cell wall observed by immunogold electron microscopy (75). The presence of this antigen may be important in eliciting the normal cell-mediated immune response, a major defense mechanism against *C. neoformans* infection in normal individuals. In this respect, *C. neoformans* MP may be functionally similar to the well-studied MP of *Candida albicans* (1). However, at present no investigations on the patterns of cytokines induced in vivo or in vitro to MP have been reported.

(iii) GalXM. Like MP, GalXM is a minor soluble antigen associated with the cell envelope of *C. neoformans* (13, 32, 70). GalXM is composed of Man, Gal, and Xyl. However, the percent composition of the monosaccharides and their associated linkages in GalXM are very different from those observed in MP. GalXM does not bind to concanavalin A because none of the mannose residues are terminally linked. This property is used to discriminate between MP and GalXM. The GalXMs from several serotypes of *C. neoformans* have been isolated and purified. Analysis of these GalXMs indicated that there is not a single molecular entity common to all serotypes. Rather, the GalXMs are a group of complex, closely related polysaccharides composed of Xyl, Man, and Gal. Nuclear magnetic resonance spectroscopy and methylation analysis have shown a minimum of 15 separate anomeric linkages. Monoclonal antibodies that are specific for GalXM have been produced (73). These monoclonal as well as polyclonal antibodies have been used to locate GalXM on the surface of *C. neoformans* Cap 67 (acapsular mutant) by immunoperoxidase, immunogold electron microscopy, and agglutination experiments (72). However, at present, the role GalXM plays in the immunobiology of *C. neoformans* is unknown.

INNATE DEFENSES

Members of the general population are commonly exposed to *C. neoformans* through inhalation of desiccated forms extant in the environment. This natural exposure rarely results in clinical symptoms associated with cryptococcosis because the innate immune resistance mechanisms present in the lungs effectively clear the yeast cells (17). However, several different lines of research indicate that the ability of phagocytic cells to clear pulmonary cryptococcal antigens is directly related to the size and composition of the polysaccharide capsule. Phagocytosis of encapsulated yeasts is inhibited by the size of the capsule (5, 41). Nevertheless, macrophages, polymorphonuclear neutrophils, and monocytes are able to phagocytize encapsulated organisms that have been opsonized with normal human serum (39). Incubation of nonencapsulated strains with normal human serum initiates both the classical and alternate complement pathways. However, opsonization of encapsulated strains is exclusively by the alternate pathway of complement activation (78). Again, the structure of capsule regulates the efficiency of clearance by phagocytic cells.

Washburn et al. (77) suggest that increased side chain substitution with D-Xyl*p* groups in the GXMs of serotypes B and C inhibits the assembly of C3bBb C3 convertase of the alternate pathway on the capsule surface. More recently, Young and Kozel (79) have demonstrated that the rate of C3 binding to serotypes B and C is significantly slower than the C3 binding rates for serotypes A and D. An inference from these investigations is that the differences in the chemical structures of the cryptococcal polysaccharide capsules influence alternate pathway complement activation, thus retarding antigen clearance and contributing to the pathogenesis of infections with certain serotypes.

However, in another study, Kozel et al. (40) found uniform iC3b deposition on phagocytosis-sensitive and phagocytosis-resistant strains with similar capsule size. These data indicate that opsonization with complement is a necessary but not sufficient requirement for phagocytosis (40). Perhaps a second signal is needed. More recently, Pfrommer et al. have reported that the uniform polysaccharide structure of the *C. neoformans* capsule allows for the efficient binding of C3B and its subsequent complete decay to C3Bi by factors H and I (57a). This observation is in agreement with the findings of Levitz and Tabuni (44) that different complement receptors are important in the phagocytosis of encapsulated and nonencapsulated strains of *C. neoformans*. Collins and Bancroft (16) have recently reported that the antiphagocytic potential of the *C. neoformans* capsule can be completely overcome by macrophages activated by the synergistic combination of tumor necrosis factor alpha and granulocyte macrophage colony-stimulating factor. Very little is known about *C. neoformans* capsule and the induction of cytokines.

SPECIFIC IMMUNE DEFENSES

The anticryptococcal effector cells of the efferent arm of the immune response are not completely known. Levitz and Dupont (43) have demonstrated that both human interleukin 2-activated T cells and NK cells are able to kill *C. neoformans* in vitro. Miller and Kohl (45) have shown that *C. neoformans* can be killed with $CD16^+$ large granular lymphocytes in vitro by antibody-dependent cell-mediated cytotoxicity, while Hidore et al. (26) have published electron photomicrographs of NK cells in direct contact with encapsulated cryptococci.

(i) Cell-mediated responses. It is generally accepted that cell-mediated immunity is the most important defense against disseminated cryptococcal infection. Both $CD4^+$ and $CD8^+$ cells have been shown to be important in clearing the yeast from the lungs (28). However, it is the $CD4^+$ cells that are responsible for DTH responses which limit the dissemination of the pulmonary infection (27). GXM has also been associated with the regulation of T-cell functions in cell-mediated responses to cryptococci. Collins and Bancroft (15) have demonstrated that the ability of encapsulated strains to inhibit phagocytosis and subsequent antigen processing and presentation by macrophages leads to a reduction of T-cell-specific,

anticryptococcal responses in vitro. Murphy et al. (54) have further shown that MP and not GXM or GalXM induces the *C. neoformans* specific DTH response. Whether *C. neoformans* MP is able to induce the production of cytokines that lead to a TH1 response, as has been demonstrated with the MP of *Candida albicans* (1), is not known. However, *C. neoformans* capsule does regulate the DTH response in an antigen-specific manner through a network of first-, second-, and third-order T suppressor cells (35, 48, 53).

The relevance of cellular immune protection in vivo to *C. neoformans* is evident in studies of both murine and human infections. Defects in cellular immunity lead to hematogenous dissemination and fatal forms of the disease in mice (28). These phenomena are also reflected in infected humans who also suffer with underlying immunodeficiencies. Cryptococcal infections are particularly serious for patients with AIDS. Cryptococcosis is the fourth most common cause of life-threatening infection in AIDS after *Pneumocystis carinii*, cytomegalovirus, and *Mycobacterium avium-Mycobacterium intracellulare* (56). Also, 10% of patients diagnosed with AIDS are also suffering from cryptococcosis (80). The distribution pattern of serotyped isolates from among these patients reveals a connection between the chemical structure of the capsule and the systemic spread of cryptococcosis in AIDS-infected individuals.

Selective infection of AIDS patients occurs with *C. neoformans* var. *neoformans* (3, 41, 62), and 99% of these are of the A serotype (34, 56). Reports from more recent investigations suggest that components of the *C. neoformans* capsule not only enhance the virulence of the yeast in immunocompromised individuals but also potentiate the infectivity of HIV in susceptible cells. During active cryptococcal infection, capsular antigens are shed and can be detected in the blood. Pettoello-Mantovani et al. (57) have reported that when GXM from serotype A was used in coculture in vitro with chronically infected H9 cells or with HIV-infected peripheral blood lymphocytes from patients with AIDS, there was a significant increase in the amount of p24 antigen as well as infectious HIV that could be detected in the supernatant. This apparent potentiation of infectivity was shown not to be secondary to increased activation of the HIV-long terminal repeat, but was more likely due to an enhancement of the binding of gp120 to CD4. Others have observed that gp120 can also potentiate the spread of cryptococcal infections (76). Bronchoalveolar macrophages treated in vitro with physiological amounts of recombinant gp120 are able to bind to encapsulated cryptococcus but not internalize the yeast. The results of these studies are consistent with the findings of Levitz and Tabuni (44) and Kozel et al. (39); they demonstrate that capsular components are necessary for the triggering of receptors on the surface of phagocytic cells for their complete fungicidal activity.

(ii) Humoral responses. Although innate and cell-mediated forces are acknowledged as the major defense mechanisms against cryptococcosis, there is much evidence that anticryptococcal antibodies can be protective (18, 19, 47). Anticryptococcal antibodies to all three antigens of the capsule have been produced. GXM has been shown to be a type 2 T-cell-independent (T-2 T-ind) antigen (4, 21, 65). Like other T-2 T-ind antigens, GXM has also demonstrated the ability to induce T-cell-dependent and T-ind mechanisms of antigen-specific tolerance (66). Currently, more information is becoming available on the effect that T-2 T-ind antigens may have on the induction of cytokines in T cells. Van Den Eertwegh et al. (74) have shown that trinitrophenyl-Ficoll will induce gamma interferon in $CD4^+$ cells. Mond and Brunswick (46) and Van Den Eertwegh et al. (74) have also shown that T-2 T-ind antigens can induce interleukin 10. These observations contribute to the speculation that the *C. neoformans* capsular polysaccharide antigens may also play a part in selecting Th1- or Th2-type T-cell responses in cryptococcal infections.

SUMMARY AND CONCLUSIONS

The major significance of the capsular polysaccharide of *C. neoformans* is its role in potentiating opportunistic infections by the yeast. It has the ability to exert a broad spectrum of influences on the immune response, from activation of phagocytic cells and complement components of the alternative pathway, to the induction of specific antibody, T-suppressor cells, DTH responses, and cytokines (51). These biological properties along with the serotype specificities are all determined by the physical properties and chemical structures of the polysaccharide antigens that compose the capsule. There is evidence not only for an association of lethal infections with serotype A in patients with advanced AIDS (34, 56), but also for a role for the capsule in directly influencing the infection of $CD4^+$ cells by HIV (57). Together, these phenomena raise intriguing questions about the possible connection between the chemistry of these capsular antigens and cryptococcal infections in AIDS patients.

One speculation is that AIDS creates the optimal physiological conditions for the establishment and spread of cryptococcosis. It has been observed that during the progression of AIDS there is a shift towards a T-2 response (14). This could lead to conditions that would inhibit the cellular immune responses that block dissemination of cryptococcal infections. Thus, an important consideration in the application of vaccine or immune modulation therapies in the treatment of cryptococcosis in AIDS victims would be the design of vaccines that could boost the T-1 immune response. It has been shown that the form and dose of an antigenic challenge can influence the induction of a T-1 or T-2 immune response (61). Recently, Murphy has reported that gamma interferon and interleukin 2 are up-regulated in the spleens of mice that produce anticryptococcal T_{DH} and T_{AMP} cells in response to immunogenic doses of cryptococcal culture filtrate antigen given with Freund's complete adjuvant (49). Perhaps purified cryptococcal antigens (e.g., MP) conjugated to an appropriate carrier or adjuvant could be used in therapeutic strategies to limit cryptococcosis in immunocompromised individuals.

Future investigations of virulence and pathogenicity in the context of defined polysaccharide antigens from encapsulated strains of *C. neoformans* will contribute to a better understanding of the regulation of cryptococcal infection and immunity at the cellular and molecular levels. This information could lead to (i) the development of effective vaccines, designed to induce not only a sufficient but also an appropriate immune response to cryptococcal antigens, and (ii) a better understanding of how these polysaccharide antigens influence T-1 and T-2 T-cell responses, how they potentiate opportunistic cryptococcosis in HIV-infected individuals, and how virulence and serotypes are related to the molecular structures of these antigens.

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